Novel Approach of Isolation of Hydroxychavicol and Checking Its Efficacy Against Polymicrobial Septicemia Infection

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of master's in biotechnology

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

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TO WHOM IT MAY CONCERN

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ACKNOWLEDGEMENT

It is with great respect and sincere gratitude that I acknowledge the invaluable support and guidance extended to me throughout the duration of this research project.

I am profoundly grateful to my supervisor, [Prof. Mayukh Bose], for their exceptional mentorship, scholarly insight, and unwavering encouragement. Their critical feedback, patience, and continuous support played a vital role in shaping the direction and quality of this work. I also wish to express my heartfelt appreciation to the faculty members and staff of the Department of Biotechnology, Sister Nivedita University, for their academic support and for providing a conducive research environment.

My sincere thanks are extended to all individuals and institutions whose contributions, directly or indirectly, have assisted me in the successful completion of this thesis. I am especially indebted to my peers and research colleagues for their constructive discussions and moral support during challenging phases of the project.

Above all, I remain eternally grateful to my family for their unwavering belief in me, constant encouragement, and personal sacrifices that enabled me to complete this academic journey.

This thesis stands as a testament to the collective support I have received, and I remain deeply appreciative of all those who contributed to its successful completion.

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ABBREVIATIONS

Serial Number	Abbreviation	Definition
1.	MIC	Minimum Inhibitory Concentration
2.	MBC	Minimum Bacterial Concentration
3.	IC50	Half-maximal Inhibitory Concentration
4.	TLC	Thin Layer Chromatography
5.	ZOI	Zone of Inhibition
6.	OD	Optical Density
7.	UV	Ultraviolet
8.	UV-Vis	Ultraviolet-Visible Spectroscopy
9.	TPP	Sodium Tripolyphosphate
10.	PDA	Potato Dextrose Agar

ABSTRACT

Polymicrobial septicemia remains a major health concern due to the increasing resistance of pathogens to conventional antibiotics. One such naturally occurring substance that has the potential to be a medication candidate is hydroxychavicol, which has low toxicity, stability, and solubility. Numerous plants, as well as certain fungi and bacteria, contain coumarin, a basic scaffold that is widely distributed in nature. This study presents a novel approach for the isolation and evaluation of hydroxychavicol, a bioactive compound extracted from *Piper betel*, and assesses its antimicrobial efficacy against bacterial and fungal strains involved in polymicrobial infections. Hydroxychavicol, a bioactive phenolic compound, was isolated using methanolic extraction followed by chloroform separation and confirmed through Thin Layer Chromatography, biochemical tests, and UV-Vis spectroscopy. A chitosan-based nanopolymer was synthesized to encapsulate hydroxychavicol, enhancing its stability and shelf life. The antimicrobial activity of the nano-formulated hydroxychavicol was assessed through Minimum Inhibitory Concentration (MIC) and IC_{50} assays against various pathogenic bacteria and fungi. Results revealed significant inhibitory effects of hydroxychavicol on microbial growth, with promising dose-dependent responses. These findings suggest that hydroxychavicol, especially in its nano-formulated state, holds potential as an effective therapeutic agent for combating polymicrobial septicemia. This study underscores the relevance of plant-derived compounds in the development of novel antimicrobial strategies. This work suggests its future application in oral healthcare, food processing or preservation, and pharmaceutical to combat rising antimicrobial resistance. Hydroxychavicol is a natural antibacterial agent that shows promise, particularly when administered by chitosan nanocarriers. Given the current period of increasing antibiotic resistance, the compounds broad-spectrum efficacy lends credence to its potential in the development of alternative treatments for illnesses. To expand its use in pharmaceuticals, food preservation, and oral healthcare items, more in vivo studies and toxicity analyses are advised.

KEY WORDS

Piper betel, Hydroxychavicol, Antimicrobial suspitability, Food poison.

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INTRODUCTION

The development of multidrug resistance in human, animal, and plant pathogens has largely resulted from the improper use of antibiotics in treating infectious diseases[1]. In recent years, common infections caused by contaminated food and oral sources have led to increasing health issues in humans. Among these, foodborne infections and associated with a higher number of infectious pathogens[2].

According to the World Health Organization (WHO), about 80% of global population relies on traditional herbal remedies to manage bacterial and fungal infections. It is now well recognized that certain plants produce and store secondary metabolites such as phenolic acids, alkaloids, flavonoids, glycosides, tannins, and essential oils which exhibit strong antimicrobial activity[3].

Piper betel Linn. is an attractive, evergreen, and perennial climbing plant known for its glossy, heart-shaped leaves, commonly referred to as betel leaves. It belongs to the Piperaceae family and typically grows to a height of 150 to 180 cm. The plant is native to India, the Philippines, Bangladesh, and Sri Lanka. In traditional India medicine, betel leaves have been widely used for various therapeutic purposes. Particularly in folk medicine, they are well known their antiseptic properties and are often applied to wounds and skin lesions to promote healing. The betel leaf is cultivated mostly in South and Southeast Asia, from India to Papua New Guinea. It needs a compatible tree or a long pole for support. Betel requires well-drained fertile soil. Waterlogged, saline and alkali soils are unsuitable for its cultivation[4]. In Bangladesh, farmers called a barouj in which to grow betel. The barouj is fenced with bamboo sticks and coconut leaves[5]. The soil is plowed into furrows of 10 to 15 m length, 75 cm in width and 75cm depth. Oil cakes, manure, and leaves are thoroughly incorporated with the topsoil of the furrows and wood ash. The cuttings are planted at the beginning of the monsoon season. Proper shade and irrigation are essential for the successful cultivation of this crop. Betel needs constantly moist soil, but there should not remain for more than half an hour[6].

Every two weeks, dried leaves are spread over the furrows, and slurry made from cow dung is sprinkled on top. It is said to be beneficial for betel development to apply various leaf types at monthly intervals. The vines will branch and grow to a height of 150 to 180 cm in three to six months. The farmer uses his right thumb to pull the leaf and its petiole to start the harvest. It takes 15 days to a month to harvest. The betel plant has found its way into the research laboratories of numerous food nutrition and chemical firms in Bangladesh.

In addition to being sold to various regions of Asia, the Middle East, Europe, and the Americas, the gathered leaves are consumed locally. In Bangladesh's rural areas, betel is a significant crop. Betel vine is another name for betel leaf. Most nations, including India, use it extensively for chewing habits in order to prevent bad breath, strengthen gums, and increase digestive fire. Betel leaves are used as a gargle mouthwash in India and as a remedy for vaginal douche in Indonesia. Betel leaf juice is used to treat skin conditions in Sri Lanka. Because of their

astringent flavor, betel leaves are roasted as well and used as cough medication[7]. Because betel leaf has an astringent taste, it is traditionally drunk in India after a meal to aid in digestion. According to ancient classical Ayurvedic writings such as Bhojankutuhalam, betel leaf is a healthy way to manage weight, promote digestion, lessen excessive coughing that accumulates in the mouth, and cleanse the throat after a meal. A betel leaf infused with therapeutic herbs is referred to as Tambul or Paan in Ayurveda. According to Ayurveda, betel leaf has a strong, sweet, bitter, alkaline, astringent, and hot flavor. Because it is an aphrodisiac and stimulates digestive heat, it kills the worms and ignites the fire of love[8].

According to a 2017 study, betel leaf includes phytocomponents with anti-inflammatory, anti-platelet, antithrombotic, antibacterial, antifungal, and antioxidant qualities. The phytocomponents hydroxyl chavicol (69.46%), 4-chromanol (24%) and eugenol (4.86%) that were identified in the betel leaf investigation have a variety of uses, including as agents[9]. Over 90 different types of betel leaf may be found worldwide, with 30 of them variations occurring in West Bengal and 45 in India. Tropical and subtropical climates are ideal for growing betel leaves. The plant's leaves are used to make dishes and spices as well as oils, fragrances, pesticides, and decorations[10]. Betel leaf is mostly used as a wrapping for chewing areca nuts, or more recently, tobacco, where it is primarily utilized to impart flavors. The oldest areca nut and crushed seashell calcium remains were discovered at the Deyong Cave archeological site in the Philippines, where the practice began about 5,000 years ago. It spread to Taiwan, South China, South Asia, and the remainder of Southeast Asia with the Austronesian migrations. Since areca nuts can be chewed on their own, it is unclear when or why betel leaves were originally mixed with them[11].

In many regions of Asia and the Pacific, chewing betel leaf is a deeply ingrained custom that dates to ancient times, according to historical documents. The mixture of components chewed with the leaf, commonly known as paan or betel quid, has experienced substantial change throughout the years, even though the practice predates the common era, with mentions dating back to the third century CE.

In the past, the quid's main ingredients were calcium hydroxide (slaked lime), areca nut (also known as supari), and catechu, a tannin-rich extract from the Acacia tree. This mixture, which had a gently stimulating impact and was thought to help with digestion and breath freshening, was significant both socially and ritually[12]. The fact that these substances were first mentioned in Indian writings as early as the ninth century CE suggests that their use and manufacture have a lengthy history.

Tobacco was not added to the betel quid mixture until much later, long into the 20th century. This signaled a change in the cultural custom. New variations of the classic chew, such as gutka—a commercially produced confection made of crushed areca nut, tobacco, and slaked lime, frequently flavored with different spices and sweeteners—arose as tobacco consumption increased. Since then, gutka has become more and more popular, particularly with younger people, whereas conventional paan has clearly declined. The move also raises public health issues, as both areca nut and tobacco are related with increased risk of swallowing malignancies.

The betel leaf itself continues to have cultural value in many South and Southeast Asian civilizations in spite of these changes. For example, betel leaves represent ceremonial purity, auspiciousness, and respect in India and Sri Lanka. On significant occasions, including weddings, religious ceremonies, and New Year's festivities, a well-organized sheaf of fresh betel leaves is traditionally offered. This offering, which can be made with cash or areca nuts, is customarily made as a token of respect and appreciation to elders, priests, astrologers, or doctors[17].

The cultural diversity around the use of betel leaf is further demonstrated by regional customs. For instance, in Bengali weddings, the bride is brought to meet the groom with her face covered in betel leaves, a custom that represents formality and modesty[18]. These customs highlight the leaf's symbolic and practical significance. The tradition persists in many forms outside of the Indian subcontinent. The inflorescence stem of the betel palm, referred to locals as "mustard stick" or "daka," is also chewed in Papua New Guinea and the Solomon Islands in addition to the leaf. Together with the leaf, this is chewed, creating a distinctive regional variation of the custom.

Numerous physiologically active phytochemicals, including beta-carotene, hydroxychavicol, vitamin E, ursolic acid, sitosterol, and eugenol, are found in betel leaves. These substances support the plant's wide spectrum of pharmacological characteristics, which include immunomodulatory, hepatoprotective, anti-ulcer, anti-inflammatory, antibacterial, antifungal, anticancer, and antidiabetic actions[13].

Phenolic compounds are generally regarded as promising therapeutic agents against a variety of conditions, such as inflammatory disorders, diabetes, cancer, cardiovascular dysfunction, neurodegenerative illnesses, and aging[14]. Hydroxychavicol is one such prevalent and significant medicinal phenolic component found in betel leaves. According to epidemiological research, hydroxychavicol has a number of biological properties, including cytotoxic, antibacterial, anti-inflammatory, anti-mutagenic, and antioxidant properties. Hydroxychavicol and other betel leaf extracts have already been shown to have antibacterial and antifungal properties against a number of common human infections. The antibacterial susceptibility of oral cavity pathogens to purified hydroxychavicol of betel leaf methanolic extract, however, has not been the subject of any research reports. Additionally, it frequently functions as a chemo-preventive agent and antioxidant[15].

A propenyl (allyl) group is joined to the benzene ring of hydroxychavicol, which also has two hydroxyl groups (OH) immediately linked to it, creating a catechol structure. In particular, HC is 4-propenyl-benzene-1,2-diol or 4-allyl-1,2-benzene-diol. 4-allylpyrocatechol is the chemical name for this catechol derivative.

reduces oxidative stress by neutralizing free radicals. demonstrates potent antifungal and antibacterial properties. efficient against bacteria such as *Candida albicans*, *Staphylococcus aureus*, and *E. coli*[16].

Stop pro-inflammatory pathways and enzymes. demonstrated in laboratory experiments to cause apoptosis, or programmed cell death, in specific cancer cells. aid in stopping DNA mutations.

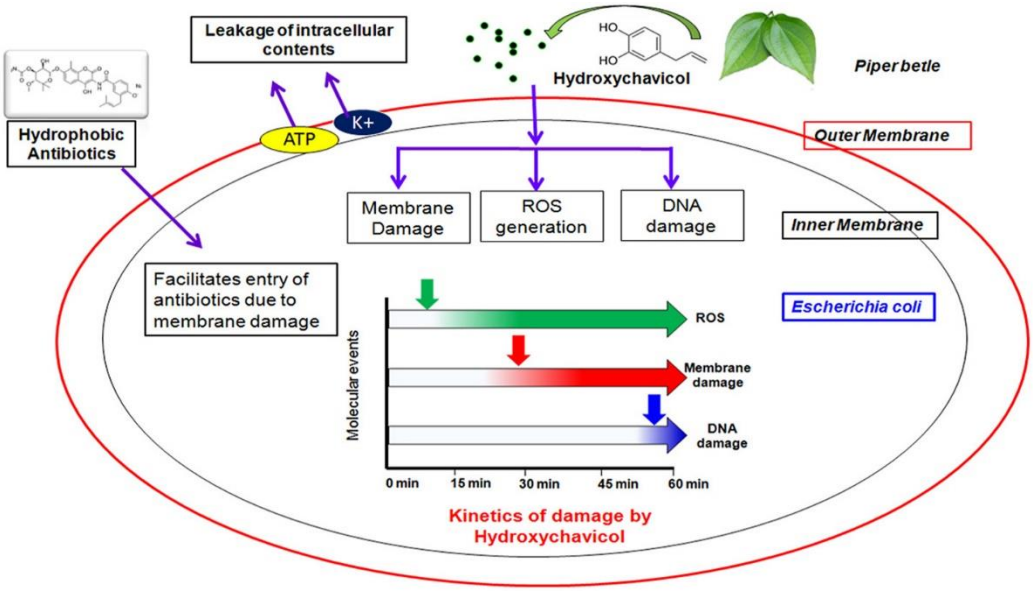


Figure 1

REVIEW OF LITERATURE

Although hydroxychavicol (HC), a primary constituent of *Piper betel* leaves, commonly known as betel vine is a key phenolic compound found in the leaves of this plant, which are widely used in South and Southeast Asia for various purposes, including culinary and medicinal applications. It is known to possess antiproliferative activity at micromolar doses on various cancer cell lines of different origins while leaving normal cell unharmed[19].

The potent antioxidant properties of hydroxychavicol are one of its most notable features. Like the body's cleanup crew, antioxidants aid in the neutralisation of dangerous free radicals, which can cause cell damage, accelerate ageing, or fuel the development of chronic illnesses like cancer and heart disease. Cellular protection is provided by hydroxychavicol, which aids the body in fending off oxidative stress. Another well-known property of hydroxychavicol is its antibacterial properties, which allow it to combat a wide range of dangerous pathogens, such

as bacteria, fungi, and even certain viruses. Because of this, it is a potentially effective natural substitute for synthetic antimicrobials, particularly at a time when antibiotic resistance is a growing global issue[20].

What's even more exciting is its potential in the fight against cancer. Early studies suggest that hydroxychavicol may help slow the growth of cancer cells and even trigger their self-destruction, a process known as apoptosis. While more research is needed, this opens the door for its use in future cancer therapies or prevention strategies[21].

Its anti-inflammatory properties provide still another significant advantage. Numerous chronic illnesses, including heart issues and arthritis, are caused by inflammation. By focusing on certain enzymes and chemical messengers in the body, hydroxychavicol has demonstrated the capacity to decrease inflammation, perhaps providing relief for those who suffer from inflammatory disorders. It's interesting to note that this chemical has a unique role in oral health[22]. It is already used in mouthwashes and toothpastes to help prevent gum disease, fight bad breath, and maintain a healthy mouth because of its capacity to destroy germs and reduce inflammation.

Furthermore, hydroxychavicol's natural origin and minimal toxicity in tests make it a safer choice for long-term use than many synthetic drugs. This is possibly one of its most comforting features.

A severe blood sickness known as polymicrobial septicemia is brought on by two or more distinct germs, most frequently bacteria. It usually results from infections in places like the gastrointestinal system, wounds, or abscesses that have a variety of microbial flora. Compared to single-organism septicemia, this condition is more dangerous and carries a larger risk of complications and death. Blood cultures and sepsis symptoms are used for diagnosis, and supportive care and broad-spectrum antibiotics are needed for therapy. With an upsurge in antibiotic resistance, plant-based antimicrobials are becoming more and more crucial in the treatment of polymicrobial septicemia[23]. Numerous plants generate bioactive substances with broad-spectrum antimicrobial properties that can combat gram-positive and gram-negative bacteria as well as fungi, including flavonoids, alkaloids, and essential oils. These substances are effective even against resistant strains because they frequently function through unusual ways (such as rupturing cell membranes or blocking enzymes). Additionally, some have antioxidant and anti-inflammatory qualities that can help control the immunological response in sepsis. They may also have synergistic effects with antibiotics, increasing the effectiveness of treatment.

With its heart-shaped leaves, piper betel is a climbing vine that grows best in warm, humid regions. Its fragrant leaves are chewed with areca nut in several Asian cultures. The plant has been prized for its medicinal qualities in addition to its traditional applications.

Bioactive substances such terpenes (like caryophyllene), alkaloids, flavonoids, and phenols (including eugenol and chavicol) are abundant in betel leaves. Strong antibacterial, antioxidant, and anti-inflammatory qualities are displayed by these phytochemicals. Because phenolic

chemicals are especially efficient against germs and fungi, piper betel is a promising natural remedy for a variety of ailments[24].

Piper betel exhibits promise as a natural antibacterial agent because of its strong phytochemicals, particularly considering the growing antibiotic resistance. It is a strong contender to help treat complicated illnesses such polymicrobial septicemia because of its broad-spectrum activity and capacity to alter immune responses.

A significant phenolic component in Piper betel, hydroxychavicol, has potent antibacterial properties against a variety of bacteria and fungi. It interferes with vital metabolic functions, damages microbial cell membranes, and prevents the production of biofilms. Because of these characteristics, it works especially well against infections caused by numerous pathogens, or polymicrobial illnesses. Its promise as a natural remedy for complex illnesses such polymicrobial septicemia is highlighted by its broad-spectrum action, low toxicity, and anti-inflammatory properties.

The severe and complex illness known as polymicrobial septicemia necessitates the use of wide and efficient antibacterial treatments. The pressing need for complementary or alternative therapies is highlighted by the rising resistance to traditional antibiotics. Because of their broad-spectrum activity, diverse modes of action, and immune-modulating qualities, plant-based antimicrobials—especially those made from medicinal herbs like Piper betel—offer encouraging promise.

One of Piper betel's main phytochemicals, hydroxychavicol, has shown potent antibacterial and anti-biofilm qualities, giving it a promising option for the treatment of polymicrobial diseases. Its efficacy against fungi and both gram-positive and gram-negative bacteria supports its use in treating multipathogen septicemia.

Prospects for the future include:

Additional in vivo and in vitro research is needed to confirm safety and efficacy.

Creation of clinically applicable formulations or standardized extracts.

Investigation of synergistic pairings with already available antibiotics to improve therapeutic results.

Clinical trials to assess efficacy in cases of septicemia in humans.

Using substances derived from plants, such as hydroxychavicol, may result in novel, practical, and long-lasting strategies for treating polymicrobial septicemia and thwarting antibiotic resistance.

MATERIALS AND METHODS

Sample Preparation

Materials And Instruments Needed:

- Fresh betel leaf
- Mortar and pestle
- Weighing balance
- Sieve
- Hot air oven
- Magnetic stirrer
- Beaker
- Filter paper
- Conical flask
- Funnel
- Separating funnel
- Hot plate

Procedure:

- Fresh Piper betel was purchased from herbarium. The fresh plant material was sent for analysis immediately, followed by washing and cutting into smaller pieces.

Then, clean-cut samples were placed in a hot air oven at 60 C –70 C until the samples dried properly for use.

- Ensure the betel leaves are fully dried.
- The betel leaves are ground into a fine powder using mortar and pestle.
- The powder is passed through a sieve for uniform particle size.
- Weigh a suitable amount of powdered betel leaves for extraction (e.g., 10 gm)

Figure 2

- Add 90ml mild hot water (40 C) to 10gm of ground betel powder on mortal pestle and again ground it until smooth paste.
- Filter the mixture with the help of a filter paper.

Figure 3

Figure 4

- After extraction the obtained extract then washed-out using chloroform with the help of a separating funnel.

- Collect chloroform containing hydroxychavicol.

Figure 5

- The chloroform containing hydroxychavicol then stir using a magnetic steroid.

- After the chloroform evaporates, a small amount of hydroxychavicol sticking to the surface of the container.

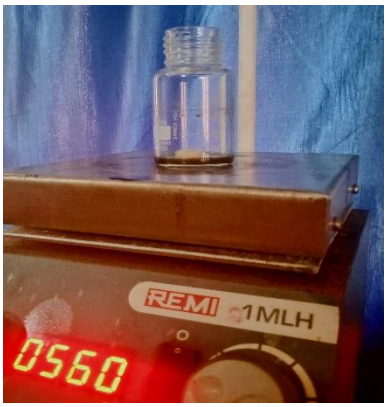


Figure 6

Figure 7

- 0.054gm hydroxychavicol obtained from 10 gm powder.

Biochemical Tests

NaOH Test

1g NaOH is added in 9ml of distilled water in 9:1 ratio then adds 2-3 drop of sample dropwise.

Sulfuric acid Test

In 2ml chloroform 1ml of sample id added shake until dissolve, the add 1ml of ethyl acetate and add sulfuric acid dropwise until the formation of red color ring.

UV-Vis Spectroscopy

The OD of the drug is taken using a spectrophotometer at the wavelengths of 280 nm.

Figure 8

Thin Layer Chromatography

Materials Needed:

- Silica
- Hydroxychavicol sample
- NaOH
- Methanol
- Small beaker
- Slide
- Pencil and ruler
- Distilled water
- Hot air oven

Procedure:

Figure 9

Figure 10

- Powdered silica is mixed with distilled water to make a gel like concentration and a put-on slide to make the TLC plates.
- These plates are then put into hot air oven so that they can dry up and become hard.
- As the TLC plates are ready, a point is marked in the middle of the plate at 1cm from the bottom using pencil and ruler. The point is known as origin point.
- A small amount of hydroxychavicol solution is applied onto the origin point using a capillary tube.
- 10ml NaOH in 90ml methanol solution is made in a beaker and the TLC plates are put in it in such a way that the origin point is dissolved in the solvent.
- The opening of the beaker is closed, and the solvent is allowed to rise until its 1 cm from the top.
- The plate is then removed, and the solvent front is marked.
- The Rf value is the calculated.

Nano Preparation

Materials needed:

- Chitosan

- Distilled water
- Acetic acid
- Sodium tripolyphosphate
- Toluene
- Magnetic stirrer
- Beaker

Procedure:

- In a beaker 10 mg of chitosan and 10 ml of distilled water are taken.
- At room temperature, the solution is stirred on a magnetic stirrer for 1 hour.
- In another beaker, 10 ml of distilled water is taken. 1 ml of distilled water is discarded, and 1 ml of acetic acid is added.
- Now this acetic acid solution is added dropwise to the chitosan solution and let it stir for 30 minutes in magnetic stirrer.
- 0.4 mg of TPP and 0.4 ml of toluene are mixed to form sodium tripolyphosphate.
- This Sodium tripolyphosphate solution is added dropwise to the chitosan solution and again stirred for another 1 hour, until it forms a homogeneous mixture.
- Filter the chitosan solution with a filter paper.

Figure 11

UV-Vis Spectroscopy

OD of the blank chitosan is measured. Peak is observed at a wavelength of 296 nm.

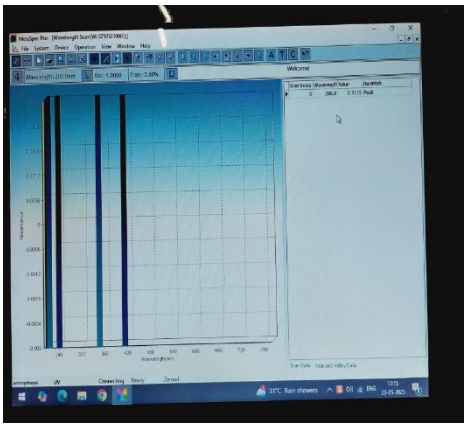


Figure 12

Coating of Drug with Nano polymer

Materials Needed:

- Pipette
- Beaker
- Measuring cylinder
- Hydroxychavicol sample
- Chitosan Nano polymer
- Magnetic Stirrer

Procedure:

- As the volume of the hydroxychavicol sample is 7 ml, same volume of nanopolymer of 7 ml is measured and taken in a beaker.
- The beaker is placed in a magnetic stirrer.
- 7 ml of hydroxychavicol solution is added dropwise with the help of pipette.
- The solution is stirred for 2 hours.

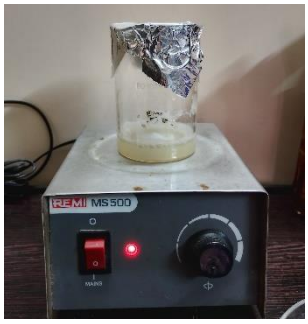


Figure 13

Stabilizing the nano-drug conjugate

After coating, the pH of the sample is raised to 4.80 for maintaining its stability.

Figure 14

UV-Vis Spectroscopy

UV-Vis spectroscopy is an analytical technique used to measure how much ultraviolet or visible light a substance absorbs. It's commonly used to analyze the chemical composition of a sample, especially in chemistry, biology and environmental science.

Preparation of Polymicrobial set

A polymicrobial set of bacteria refers to a mixture of multiple types of microorganisms, often including different species or strains of bacteria. Here, set 1 and set 2 polymicrobial set are performed.

In Set 1 microorganisms include: *Enterococcus faecalis*, *Listeria monocytogens*, *Serratia marcescens*.

In Set 2 microorganisms include: *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Listeria monocytogenes*.

Culture plate preparation

- Preparation of LB media: For 10ml the composition of LB media include- Tryptone (0.1g), Yeast Extract (0.05g), Sodium Chloride (0.1g), Agar (0.15g).
- After heat the agar media is ready to use.
- Pour the agar media in Petri dish inside the laminar air flow, wait until solidify.

- After solidification spread each bacterial strain in individual plate using a sterile glass rod or spreader as marked accordingly.

- The plate is then incubated for overnight under appropriate conditions to allow for the growth of isolated colonies.

Polymicrobial set

- LB broth is prepared as per quantity.

- Inoculate each microorganism individually.

- When all strains individual OD reached within a certain range of 0.6 then again, another LB broth is prepared.

- The individual liquid cultures then add to the newly prepared broth according to the Set mentioned.

- Then again when the OD of the polymicrobial broth reached to 0.6, MIC/IC50 is performed.

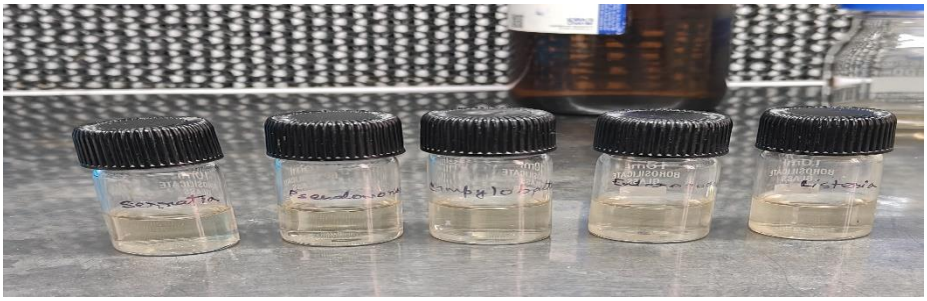


Figure 15

MIC of Bacterial Polysets

MIC of polyset 1 and polyset 2 are done to check if the drug sample can inhibit the growth of bacterial colonies. Dose calculation is done based on the concentration and various doses are given to find out its MIC.

Figure 16

Procedure

- When the standard's OD reached within a certain range (0.56 - 0.62), MIC is performed.
- 3 mL of standard solution is taken in a falcon.
- A few volumes of the standard solution are discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
- Then the drug dose is to be added to the falcon and the falcon is placed on a shaker.
- OD readings are taken at an interval of 1 hour.

IC50 of Bacterial Polysets

IC50 of bacteria refers to the concentration of the drug required to inhibit 50% of bacterial growth or activity. IC50 of polyset 1 and polyset 2 are done to find out the concentration of the drug sample where there is 50% reduction in the growth and activity of bacterial colonies. Dose calculation is done based on concentration and various doses are given to find out its IC50.

Procedure:

- When the standard's OD reached within a certain range (0.56 - 0.62), MIC is performed.
- 3 mL of standard solution is taken in a falcon.

- A few volumes of the standard solution are discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
- Then the drug dose is to be added to the falcon and the falcon is placed on a shaker.
- OD readings are taken at an interval of 1 hour.

ZOI of Bacterial Polysets

The Zone of Inhibition is the area around an antimicrobial agent where the growth of microorganisms is inhibited.

Procedure:

- Prepare LB media and pour into Petri dish.
- Allow the agar to solidify.
- Spread the bacterial suspension using a spreader over the entire surface of the agar plate.
- Use a sterile cork borer to punch well in the agar plate.
- Remove the agar plug carefully to create a clean well.
- Add 100µL of sample into the well.
- Allow the plates to sit at room temperature for 30-60 minutes to let the agent diffuse into the agar.
- Incubate the plates at 37C for overnight.

PDA Media preparation for Fungus Culture

The composition of PDA media for 1000ml include – 200g Potato, 20g Dextrose, 20g Agar, Distilled water 1L.

Procedure:

- Boiled diced potatoes in distilled water, then strain the mixture.
- Add dextrose and agar to the potato extract.

- Sterilize the mixture by autoclaving at 121 C for 15-20 minutes.
- Pour the sterilized PDA medium into sterile petri plates.
- Allow the medium to solidify.
- Use a sterile spreader to spread the given fungus strain evenly spread across the plate.
- Incubate the plate at suitable temperature for 3-5 days or until fungal growth is visible.

Figure 17

MIC of Fungus (*Aspergillus niger)*

The Minimum Inhibitory Concentration of a fungicide or antifungal agent in the lowest concentration that inhibits visible growth of a fungus.

Procedure:

- Fungus culture is inoculated in the LB broth, and when the standard's OD reached within a certain range (0.56 - 0.62), MIC is performed.
- 3 mL of standard solution is taken in a falcon.
- A few volumes of the standard solution are discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
- Then the drug dose is to be added to the falcon and the falcon is placed on a shaker.
- OD readings are taken at an interval of 1 hour.

IC50 of Fungus (*Aspergillus niger)*

The IC_{50} (half maximal inhibitory concentration) of a fungal culture refers to the concentration of a substance required to inhibit 50% of fungal growth or activity under specific experimental conditions. The lower the IC_{50}, the more potent the antifungal agent. It's commonly used in pharmacology, microbiology, and toxicology to assess the efficacy of antifungal agents against fungal strains.

Procedure:

- Fungus culture is inoculated in the LB broth, When the standard's OD reached within a certain range (0.56 - 0.62), MIC is performed.
- 3 mL of standard solution is taken in a falcon.
- A few volumes of the standard solution are discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
- Then the drug dose is to be added to the falcon and the falcon is placed on a shaker.
- OD readings are taken at an interval of 1 hour.

ZOI of Fungus (*Aspergillus niger)*

The Zone of Inhibition is the area around an antimicrobial agent where the growth of microorganisms is inhibited.

Procedure:

- Prepare LB media and pour into Petri dish.
- Allow the agar to solidify.
- Spread the fungal suspension using a spreader over the entire surface of the agar plate.
- Use a sterile cork borer to punch well in the agar plate.

- Remove the agar plug carefully to create a clean well.
- Add 100µL of sample into the well.
- Allow the plates to sit at room temperature for 30-60 minutes to let the agent diffuse into the agar.
- Incubate the plates at 37C for overnight.

RESULT AND DISCUSSION

Thin Layer Chromatography

Distance travelled by the solvent = 6.5 cm

Distance travelled by the solute = 4 cm

Therefore, the Rf value = (Distance travelled by the solute / Distance travelled by the solvent)

= 4 / 6.5

= 0.62

The standard Rf value of hydroxychavicol is 0.64, so closer to that so it confirms that the drug is hydroxychavicol.

Biochemical Tests

NaOH Test

Experiment	Observation	Result
1g NaOH is added in 9ml of distilled water in 9:1 ratio then adds 2-3 drop of sample dropwise.	Formation of orange color.	Presence of hydroxychavicol

Table 1

Figure 18

Sulfuric acid Test

Experiment	Observation	Result
In 2ml chloroform 1ml of sample id added shake until dissolve, the add 1ml of ethyl acetate and add sulfuric acid dropwise until the formation of red color ring.	Formation of red color ring.	Presence of hydroxychavicol.

Table 2

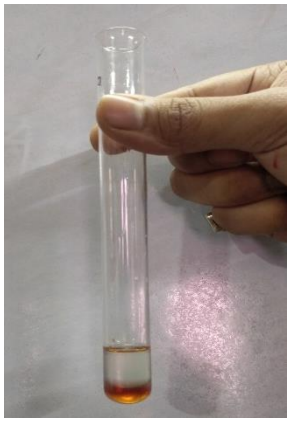


Figure 19

UV-Vis Spectroscopy

Wavelength	OD
280 nm	0.048

Table 3

Calculation of sample concentration

Weight of sample = 0.054 g

Volume of sample = 10 ml

Therefore, concentration = 5.4 mg/ml = 2.7 mg/ml = 2700 µg/mL

MIC of Bacterial Culture

Set 1

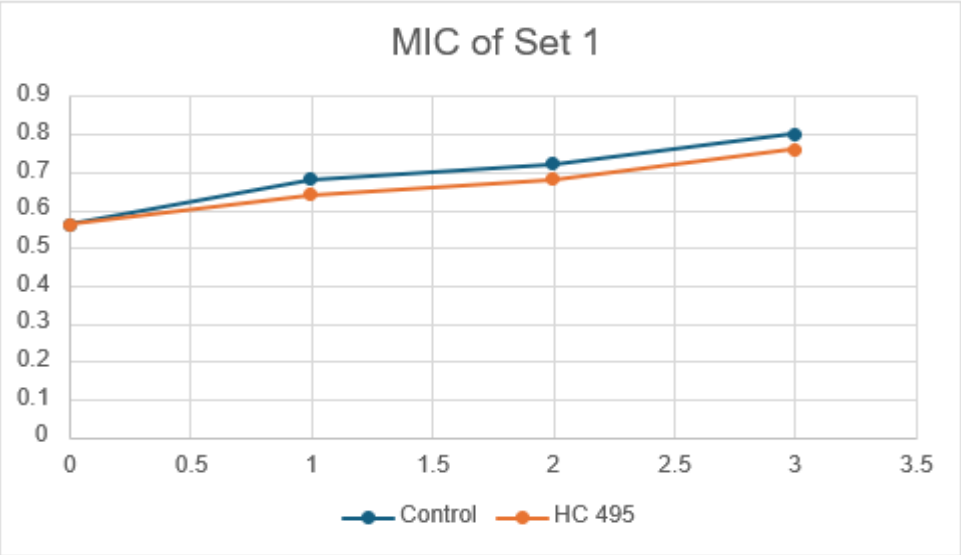
C1 = 2700 µg/mL, C2 = 495, V2 = 3 mL

V1 = 495 × 3 / 2700 = 0.55 mL = 550 µL

Starting OD = 0.56 Dose = 550 µL Time interval = 1 hour

Control	HC 495
0.68	0.64
0.72	0.68
0.80	0.76

Table 4



Set 2

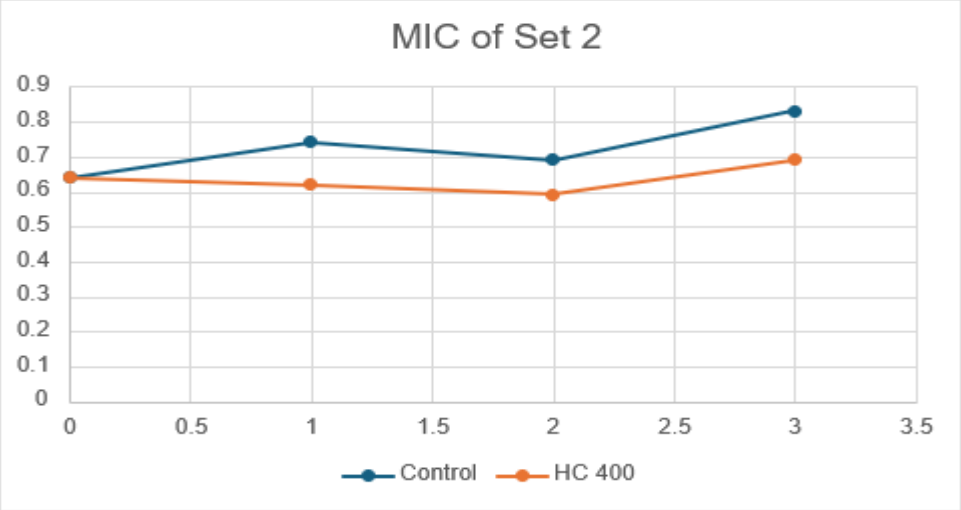
C1 = 2700 µg/mL, C2 = 400, V2 = 3 mL

V1 = 400 × 3 / 2700 = 0.44 mL = 440 µL

Starting OD = 0.64 Dose = 440 µL Time interval = 1 hour

Control	HC 400
0.74	0.62
0.69	0.59
0.83	0.69

Table 5



IC50 of Bacterial Culture

Set 1

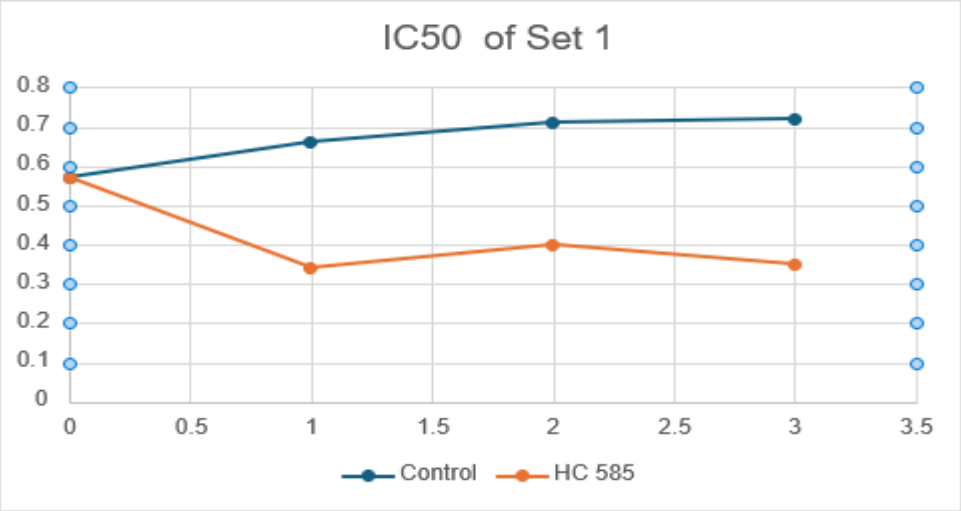
C1 = 2700 µg/mL, C2 = 585, V2 = 3 mL

V1 = 585 × 3 / 2700 = 0.65 mL = 450 µL

Starting OD = 0.57 Dose = 450 µL Time interval = 1 hour

Control	HC 585
0.66	0.34
0.71	0.40
0.72	0.35

Table 6



Set 2

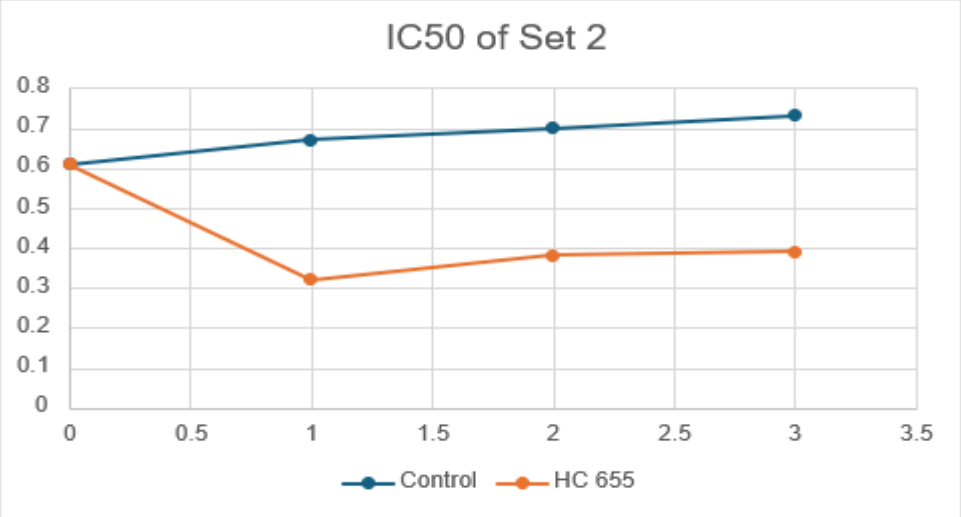
C1 = 2700 µg/mL, C2 = 655, V2 = 3 mL

V1 = 655 × 3 / 2700 = 0.73 mL = 727 µL

Starting OD = 0.61 Dose = 655 µL Time interval = 1 hour

Control	HC 655
0.67	0.32
0.70	0.38
0.73	0.39

Table 7



ZOI of Bacterial Polyset

Set 1

Diameter 1 = 3 cm
Diameter 2 = 2.8 cm
Diameter 3 = 2.9 cm

Average Diameter = (3+2.8+2.9) / 3 = 2.9 cm
Well Diameter = 1.8 cm

ZOI (Zone of Inhibition) = (2.9-1.8) = 1.1 cm

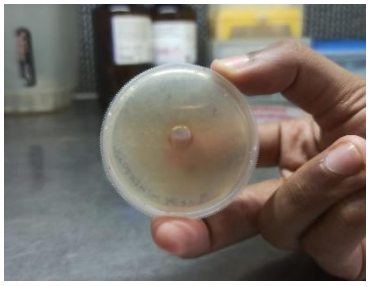


Figure 20

Set 2

Diameter 1 = 2.7 cm
Diameter 2 = 2.8 cm
Diameter 3 = 2.9 cm

Average Diameter = (2.7+2.8+2.9) / 3 = 2.8 cm
Well Diameter = 1.9 cm

ZOI (Zone of Inhibition) = (2.8-1.9) = 0.9 cm

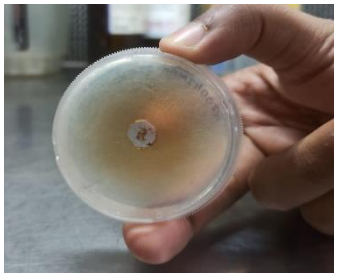


Figure 21

MIC of Fungal Culture

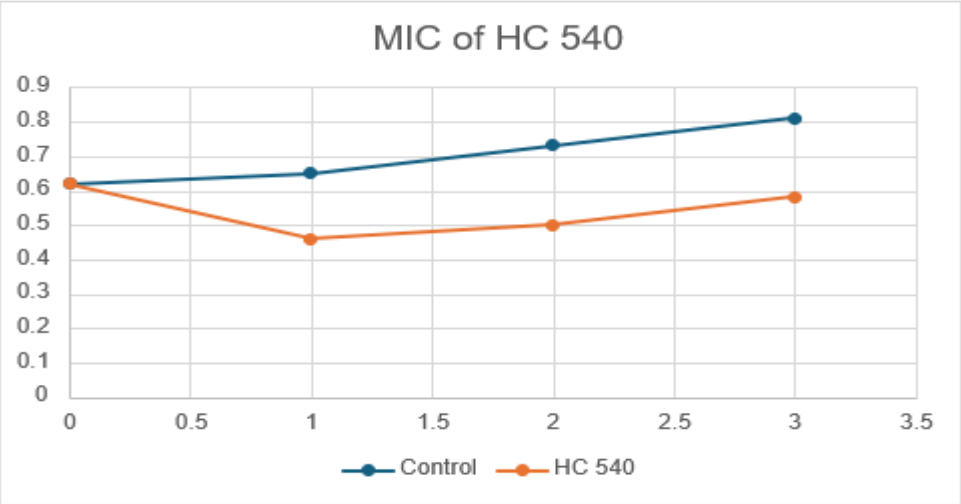
C1 = 2700 µg/mL, C2 = 540, V2 = 3 mL

V1 = 540 × 3 / 2700 = 0.6 mL = 600 µL

Starting OD = 0.62 Dose = 600 µL Time interval = 1 hour

Control	HC 540
0.65	0.46
0.73	0.50
0.81	0.58

Table 8



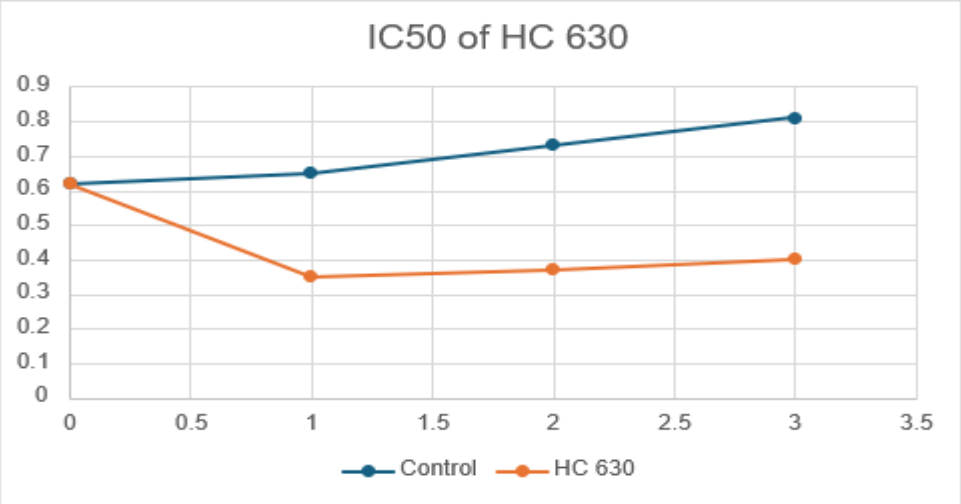
IC50 of Fungal Culture

C1 = 2700 µg/mL, C2 = 630, V2 = 3 mL

V1 = 630 × 3 / 2700 = 0.7 mL = 700 µL

Starting OD = 0.62 Dose = 700 µL Time interval = 1 hour

Control	HC 630
0.65	0.35
0.73	0.37
0.81	0.40



ZOI of Fungal Culture

Figure 22

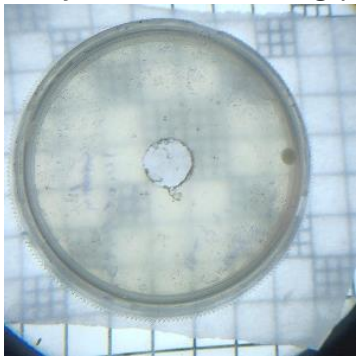
Diameter 1 = 3 cm
Diameter 2 = 2.8 cm
Diameter 3 = 2.9 cm

Average Diameter = (3+2.8+2.9) / 3 = 2.9 cm
Well Diameter = 0.8 cm

ZOI (Zone of Inhibition) = (2.9-0.8) = 2.1 cm

CONCLUSION

This study effectively extracted and characterized hydroxychavicol, a strong phenolic component from *Piper betel* leaves, and assessed its antimicrobial activity against polymicrobial bacterial and fungus strains typically linked to oral and foodborne illnesses. Hydroxychavicol was validated by means of a combination of biochemical assays, thin-layer chromatography (Rf = 0.62), and UV-Vis spectroscopy.



Using chitosan in subsequent nanoparticle formulation greatly improved the hydroxychavicol potential delivery and stability. By means of MIC, IC50 and ZOI tests, the nanodrug conjugate showed rather strong antibacterial activity. With quantifiable dose-dependent decreases in optical density and prominent zones of inhibition, the molecule demonstrated effective inhibitory action against both bacterial strains and the fungal strain.

These results support the therapeutic possibilities of hydroxychavicol produced from *Piper betel,* especially in its nanoform, as a natural antibacterial agent. In future this study may focus on in vivo efficacy, toxicological assessment and as a food preservative. The study emphasizes a promising avenue in plant based antimicrobial medication development.

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